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The fragile X-related proteins FXR1P and FXR2P contain a functional nucleolar-targeting signal equivalent to the HIV-1 regulatory proteins

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Fragile X syndrome is caused by the absence of the fragile X mental-retardation protein (FMRP). FMRP and the fragile X-related proteins 1 and 2 (FXR1P and FXR2P) form a gene family with functional similarities, such as RNA binding, polyribosomal association and nucleocytoplasmic shuttling. In a previous study, we found that FMRP and FXR1P shuttle between cytoplasm and nucleoplasm, while FXR2P shuttles between cytoplasm and nucleolus. The nuclear and nucleolar-targeting properties of these proteins were investigated further. Here, we show that FXR2P contains in its C-terminal part, a stretch of basic amino acids 'RPQRRNRSRRRRFR' that resemble the nucleolar-targeting signal (NoS) of the viral protein Rev. This particular sequence is also present within exon 15 of the *FXR1* gene. This exon undergoes alternative splicing and is therefore only present in some of the FXR1P isoforms. We investigated the intracellular distribution of various FXR1P isoforms with (iso-e and iso-f) and without (iso-d) the potential NoS in transfected COS cells treated with the nuclear export inhibitor leptomycin-B. Both iso-e and iso-f showed a nucleolar localization, as observed for FXR2P; iso-d was detected in the nucleoplasm outside the nucleoli. Further, when a labelled 16-residue synthetic peptide corresponding to the NoS of FXR1P was added to human fibroblast cultures a clear nucleolar signal was observed. Based on these data we argue that the intranuclear distribution of FXR2P and FXR1P isoforms is very likely to be mediated by a similar NoS localized in their C-terminal region. This domain is absent in some FXR1P isoforms as well as in all FMRP isoforms, suggesting functional differences for this family of proteins, possibly related to RNA metabolism in different tissues.

INTRODUCTION

The absence of the *FMR1* gene product is responsible for the fragile X syndrome, which is characterized mainly by mental retardation and macro-orchidism (1–4). The first functional motifs identified in the fragile X mental-retardation protein (FMRP) were two hnRNP K-homologous (KH) domains and an RGG box, both thought to mediate RNA binding (5–7). Indeed, FMRP binds RNA *in vitro* (6–9) and is associated with ribosomes in an RNA-dependent manner (10–13). In addition, FMRP contains a nuclear localization signal (NLS) and a nuclear export signal (NES) (14–16). The NES of FMRP is similar to the leucine-rich NES of the Rev/PKI type, which is present in many nucleocytoplasmic shuttling proteins.

Two autosomal homologues of FMRP, called FXR1P and FXR2P (fragile X-related proteins 1 and 2), have been identified and studied (17–19). The three proteins (FXR proteins) are highly homologous. Consistent with this, the FXR proteins share both motifs (NLS, KH domains, NES and RGG box) and functional features, including RNA-binding ability (17,18,20) and ribosome association (12,13,17,21). The FXR proteins can interact with themselves, with each other, both *in vitro* and in an over-expressed cellular system (18,22,23). However, a recent study shows that the FXR proteins preferentially form homo-multimers under physiological conditions in mammalian cells, suggesting that they participate in mRNP particles with separate function (23). This is supported by the intracellular distribution of the different proteins in different tissues. In brain, the three proteins are co-expressed in the cytoplasm of neurons. In testis, FMRP is expressed mainly in spermatogonia, FXR1P in post-meiotic spermatids and FXR2P in all testicular cells (19,24). Moreover, FXR1P is highly expressed in muscle and heart, where FMRP is almost absent (19,25). Recent studies showed that, like the *FMR1* gene, the *FXR1* gene is alternatively spliced. At least seven spliced forms (iso-a to iso-g) have been identified (26). In contrast to FMR1, the mechanism of alternative splicing appears to be tissue specific for *FXR1*, with different isoforms expressed preferentially in different tissues (25).

Although the FXR proteins are detected predominantly in the cytoplasm, they can shuttle between cytoplasm and

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nucleus. The nuclear export of the FXR proteins is mediated by the export receptor exportin1, since its functional inhibition by Leptomycin B (LMB) results in the nuclear accumulation of the FXR proteins (27). In addition, FMRP shuttles between cytoplasm and nucleoplasm whereas FXR2P shuttles between cytoplasm and nucleolus (27).

In an attempt to trace functional differences between the FXR proteins, we focused our attention on their C-terminal regions, which are highly divergent, sharing only 6% similarity (18). We identified and functionally characterized a specific nucleolar-targeting signal (NoS) in both FXR2P and some FXR1P isoforms. This finding suggests that the presence or absence of this new functional motif determines the shuttling of the FXR proteins to different nuclear compartments.

RESULTS

FXR1P and FXR2P have functional homologies with the NoS of the Rev protein

FMRP and FXR2P are very homologous in their N-terminal portions, but highly divergent in their C-termini. Since after nuclear export inhibition FXR2P is found in the nucleolus, while FMRP is excluded from it, we hypothesized that the unique C-terminal tail of FXR2P might provide a specific signal for nucleolar targeting. To examine this, we aligned the amino acid sequences of the two proteins in the C-terminal region. We identified two stretches of ~10 amino acids, $_{546}$RRRRSRRRR$_{554}$ (NoS1) and $_{582}$RPQRRNRSRRRRNR$_{595}$ (NoS2), in FXR2P that were enriched in the basic amino acid arginine. Neither of these sequences is present in any of the known FMRP isoforms. We designated these sequences NoS1 and NoS2 since they showed a striking similarity to two known NoSs identified in the Rev protein of the human immunodeficiency virus type 1 (HIV-1) (Fig. 1) and the Rex protein of the human T cell leukaemia/lymphoma virus type I (HTLV-I) (28). When fused to β-galactosidase, the NoS of Rev is capable of autonomously directing the hybrid protein to the cell nucleolus (28,29). Therefore, we hypothesized that those stretches of basic amino acids might be critical also for the nucleolar localization of FXR2P.

The molecular characterization of the spliced variants of the *FXR1* gene (iso-a to iso-g) revealed amino acid sequence similarities to the C-terminal tail of FXR2P for some *FXR1* isoforms (iso-e to iso-g) (26). All the FXR1P isoforms contain the first basic stretch (NoS1) present in FXR2P (Fig. 2), but, as a consequence of alternative spicing of exon 15, only three of the seven FXR1P isoforms (iso-e, iso-f and iso-g), also contain the NoS2 motif (Fig. 2).

We produced a synthetic peptide, C-PQRRNRSRRRRFRGQ, encompassing the NoS2 of FXR1P. The peptide was bound to a fluorescent dye (Cy-3) and added to the medium of fibroblast cultures. By using immunofluorescence on fixed cells, we clearly detected that the peptide was targeted to the nucleoli (Fig. 3). No nucleolar or cellular uptake was found with an unrelated fluorescent peptide (data not shown).

The overall sequence data and our *in vivo* peptide targeting experiments led us to conclude that in the C-terminus of FXR1P and FXR2P there are indeed sequences (NoS1 and NoS2) that can function as NoS. Interestingly, the second of these new FXR protein domains (NoS2) is isoform specific for FXR1P (Fig. 2).

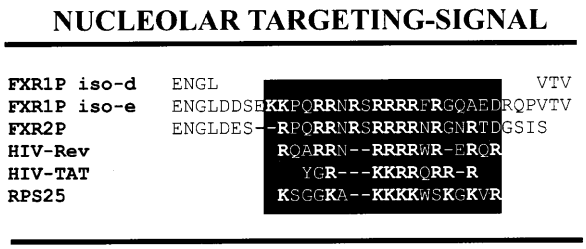

Figure 1. Amino acid sequence comparison and alignment of the potential NoS (NoS2) in FXR1P and FXR2P and described functional NoS in HIV-REV, HIV-TAT and the ribosomal protein RPS25

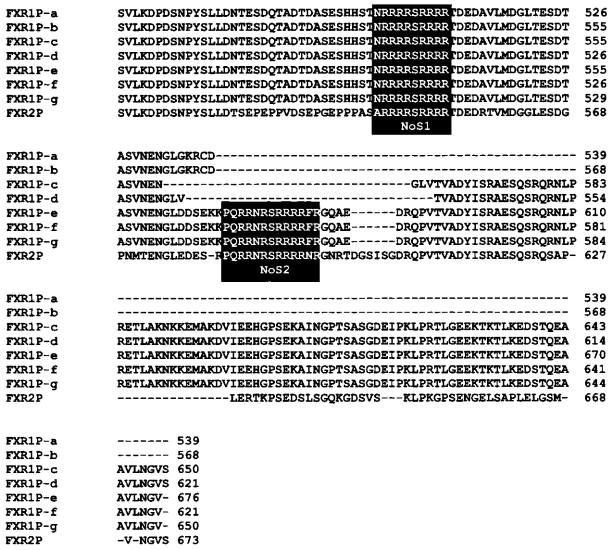

Figure 2. Amino acid sequence alignment of the C-terminal parts of the FXR1P iso-a to iso-g and FXR2P. The potential NoS (NoS1 and NoS2) present in FXR1P iso-a to iso-g and FXR2P are highlighted.

FXR1P iso-e and iso-f shuttle between cytoplasm and nucleolus

Next, we determined whether the NoSs identified in FXR1P were functional in the context of the entire protein, as shown previously for FXR2P. The direct approach was to analyse the intracellular distributions of naturally occurring FXR1P isoforms with (iso-e and iso-f) and without (iso-d, previously called FXR1P long isoform) the NoS2. Notably, iso-f and iso-d are identical except for the 27 amino acids encoded by exon 15.

We cloned the full-length open reading frames of murine *Fxr1h* iso-e and iso-f and human *FXR1* iso-d in a mammalian expression vector and individually transfected the constructs into COS cells. The expression patterns of the different isoforms were analysed by western blotting and immunofluorescence, using specific anti-FXR1P antibodies (Ab 2107). At 24 and 48 h after transfection, an exclusively cytoplasmic localization for the three isoforms was observed (Fig. 4c, f and h). We showed recently that the activity of a leucine-rich NES

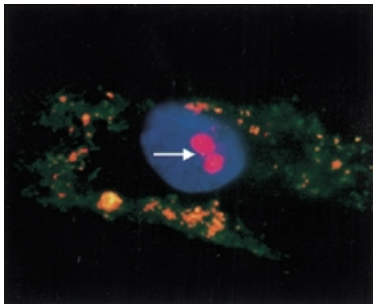

Figure 3. A fluorescently labelled synthetic peptide containing the NoS, NoS2, as found in FXR1P iso-e to iso-g, was added to the medium of cultured fibroblasts. After 24 h the fluorescent-stained peptide was found in the nucleoli (indicated by the arrow).

is necessary to maintain the cytoplasmic localization of FMRP, FXR1P iso-d and FXR2P, and that LMB, an inhibitor of nuclear export, interferes with this distribution (27). The expression of FXR1P iso-d, iso-e and iso-f was investigated further on transfected COS cells after LMB treatment. After 15 h LMB treatment (50 ng/ml), the localization pattern of iso-e was either cytoplasmic only, or cytoplasmic and nucleolar (Fig. 4g), which was comparable to that seen for FXR2P (Fig. 4b). Often the FXR1P iso-e signal was seen clearly at the periphery of the nucleolus, as reported also for FXR2P. After LMB treatment, we could also detect FXR1P iso-f in the nucleolus (Fig. 4i), although in fewer cells in comparison with FXR1P iso-e. This difference might be due to the lack of sequence from exon 12 in FXR1P iso-f. A heterogeneous localization pattern of FXR1P iso-d was evident after LMB treatment, consisting of: (i) only cytoplasmic; (ii) cytoplasmic and nucleoplasmic; (iii) only nucleoplasmic (Fig. 4d). Transfected cells expressing FXR1P iso-d (or FMRP, data not shown) in the nucleus consistently showed absence of staining in their nucleoli, which were visible under the microscope as dark spots (Fig. 4d). As indicated by DAPI staining (Fig. 4e and j), a nucleolar staining for FXR1P and FXR2P is detected only when a mature nucleolus appears.

These results indicate that some isoforms of FXR1P shuttle between cytoplasm and nucleolus, like FXR2P, and others between cytoplasm and nucleoplasm, like FMRP. Moreover, the second stretch (NoS2) is likely to be the functional NoS of the FXR proteins, since the NoS1 alone is not able, or sufficient, to target FXR1P iso-d to the nucleolus.

FXR1P iso-e is expressed solely in heart and skeletal muscle

Previous studies by RNA *in situ* hybridization as well as immuno-histochemistry indicated that the expression pattern of FXR1P is remarkably different to that of FMRP (24,30). We extended this expression analysis using western blotting, which enables the analysis of each individual FXR protein isoform. Although the expression level of FMRP varied between different human tissues, such as kidney, liver, brain, heart and skeletal muscle, the ratio of the expressed isoforms per tissue seemed to be comparable (Fig. 5B). In contrast, the individual FXR1P isoforms were differentially expressed in these tissues. Skeletal muscle and heart exhibited a unique long FXR1P isoform (Fig. 5A). These results independently confirm the findings of Khandjian *et al.* (25), which were obtained using a different antibody (Ab 2107).

Available data indicate variations in the levels of different *FXR1* splice variants in different tissues. Particularly, more *FXR1* iso-e has been found in heart than in any other tissue by RT–PCR (26). To determine which FXR1P protein isoform(s) are expressed in heart (and skeletal muscle), COS cells were transfected with expression vectors containing FXR1P iso-d, iso-e and iso-f (Fig. 5C). After SDS–PAGE and western blotting the molecular masses of the transiently expressed isoforms were compared with the isoforms expressed in heart (Fig. 5C). These results show that the major form detected in heart and skeletal muscle corresponds to FXR1P iso-e.

DISCUSSION

FMRP, FXR1P and FXR2P are members of a novel family of RNA binding proteins. At steady state the main localization of

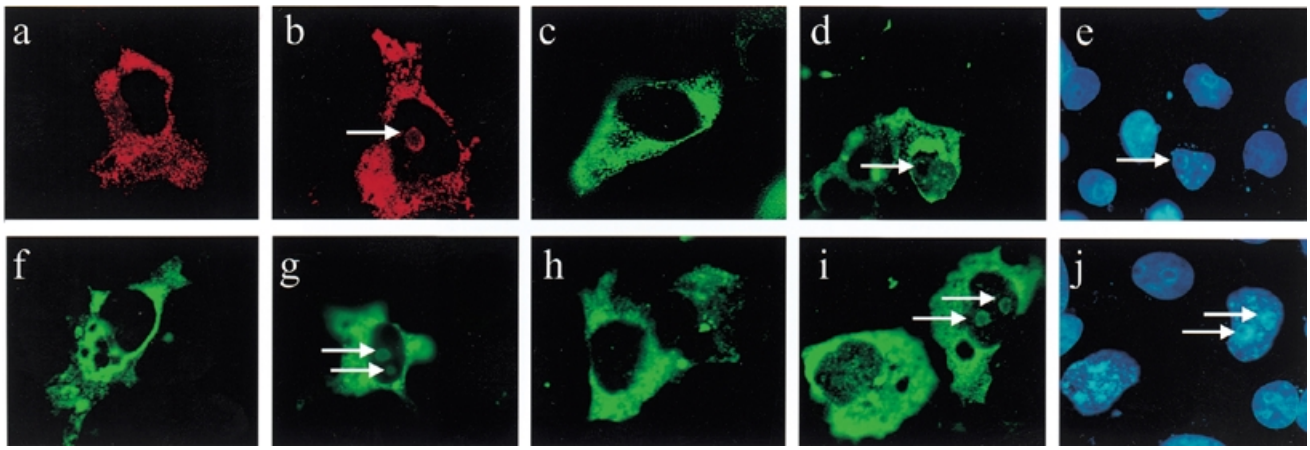

Figure 4. COS cells transfected with expression plasmids encoding FXR2P (**a** and **b**), FXR1P iso-d (**c**–**e**), FXR1P iso-e (**f** and **g**) and FXR1P iso-f (**h**–**j**). The encoded proteins were visualized after transfection using the rabbit polyclonal antibody 1937 against FXR2P (a and b) and anti FXR1P (c, d and f–i). The subcellular localization of the FXR1P and FXR2P proteins was examined after transfection followed by overnight treatment with LMB (b, d, g and i). DAPI staining of nuclei (e and j).

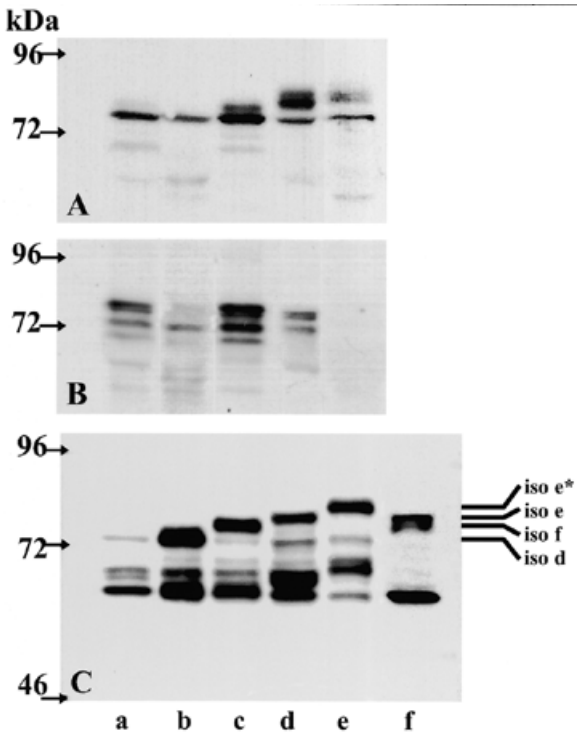

Figure 5. Human kidney (a), liver (b), brain (c), heart (d) and skeletal muscle (e) were analysed for the presence of FXR1P (**A**) and FMRP (**B**). The proteins were separated by SDS–PAGE followed by western blotting. After the detection of FXR1P with AB2107 (A) the nitrocellulose was stripped and re-incubated with AB1C3 for the detection of FMRP (B). (**C**) COS cells transfected with different FXR1P isoforms. Lane a, COS not transfected; lane b, COS transfected with FXR1P iso-d; lane c, COS transfected with FXR1P iso-f; lane d, COS cells transfected with iso-e; lane e, COS cells transfected with a flagged iso-e; lane f, heart. The bands at 60–65 kDa are endogenous FXR isoforms from the COS cells.

these proteins is in the cytoplasm where they associate with ribosomes (10–13,20). However, nucleocytoplasmic shuttling has been demonstrated and shown to depend on the presence of a functional NLS and NES in these proteins (14,15,27). Inactivation of the NES-mediated nuclear export pathway, either by introducing mutations in the NES or by direct inhibition with LMB, shifts the localization of FMRP to the nucleoplasm (15,16,27), while FXR2P was found more exclusively in the nucleolus (27).

Here we describe the identification of a functional NoS (NoS2) in FXR2P that may explain the reported distinct nuclear targeting of FXRP2 and FMRP.

Since FMRP lacks this nucleolar signal, the occasional localization in the nucleolus (10) must be a result of other mechanisms, perhaps by interaction with other nuclear proteins. A direct binding of FMRP with NUFIP (nuclear FMRP-interacting protein) (31) has been demonstrated. Furthermore, binding of FMRP to a complex that includes FXR1P, FXR2P and nucleolin has been found (22). Like FMRP, nucleolin does not contain a NoS, but is able to bind to other nucleolar components, such as rRNA (32). Another explanation for the occasional nucleolar FMRP could be that one of the FMR1 isoforms (33) has an alternative localization in the nucleolus. However, only one FMR1 splice form (ISO12) has been found with a different localization; this is due to the splicing out of exon 14, which contains the coding sequence for NES. FMRP ISO12 has been extensively studied and has been found in the nucleoplasm with the exclusion of the nucleolus (27,34).

Like *FMR1*, *FXR1* is also alternatively spliced. All of the individual FXR1P isoforms contain the first basic stretch (NoS1), and at least three isoforms, iso-e, iso-f and iso-g, contain the NoS2 sequence, due to alternative splicing of *FXR1* exon 15. This finding, together with the nucleolar localization of FXR1 iso-e and iso-f, indicates that NoS2 is very likely the main functional NoS, even if both NoS1 and NoS2 cooperate in determining the nucleolar targeting. In contrast to FMRP, the individual FXR1P proteins display a specific tissue distribution. In particular, FXR1 iso-e and iso-f are found almost exclusively in heart and skeletal muscle (Fig. 5) (25,26). This tissue specificity and the fact that FXR1P isoforms (iso-e to iso-g) contain functional NoSs, suggest a tissue-specific function for the different isoforms.

Notably, the NoS2 motifs of FXR2P and FXR1P are almost identical to the NoS found in the Rev protein of HIV-1, the Rex protein (one of the regulatory genes from HTLV-I), the HIV-TAT protein and the ribosomal RPS25 protein (28,35,36). Apart from the NoS2, the conservation of functional domains between the FXRs and the Rev protein is remarkable, as they all contain a NLS, a leucine-rich NES and an RNA-binding domain. HIV-1 Rev is a 116 amino acid nucleocytoplasmic shuttling protein with a predominant localization in the nucleolus (37,38). Rev binds to a specific target on viral unspliced and singly spliced RNAs via the unique Rev response element (RRE). The nuclear export of this RNA to the cytoplasm is then mediated by binding of the Rev NES to the nuclear export receptor CRM1 (exportin1) and is dependent on the GTP-bound form of the cellular RAN protein (39–42). The importance of the NoS for the correct function of Rev, nucleolar targeting and unique RNA binding, has been demonstrated (28,43). The similarities described between the FXR proteins, the Rev protein and some ribosomal proteins suggest that the FXR proteins might perform similar functions. These may include the ability to interact with sequence- or structure-specific RNA, regulate their own translation through binding to their own RNA (44–46) and follow an exportin1 and GTP/RAN-mediated nuclear export pathway (41,42). Indeed, binding of FMRP to its own RNA has been demonstrated (8,47); however, whether the FXR proteins also regulate their own expression has yet to be studied.

Another interesting finding is that FXR1P, like many ribonucleoproteins, including nucleolin, can function as an autoantigen (48–50). Using sera from patients suffering from scleroderma an autoantigen was isolated that was identified as FXR1P. However, when screening the sera of the patients by immunofluorescence, the observed strong nucleolar staining could not be explained by the authors (49). The finding that the FXR1P splice products iso-e to iso-g contain a functional NoS suggests that the antibodies in the sera of these patients were directed against one of those FXR1 isoforms.

In addition to possible nucleolar targeting, it has been demonstrated that the NoS from HIV-TAT can function as a carrier too, making it possible to transport not only HIV-TAT, but also artificial fusion proteins (NoS–β-galactosidase), across several membranes including the blood brain barrier (35,51). This specific membrane binding and cellular uptake, mediated by a nucleolar signal motif, has also been observed to

be the mechanism by which the parathyroid hormone-related protein is taken up and targeted (52). An intriguing question is whether the strong boosting of the immune response seen in patients with scleroderma, leading to the autoimmune process, is caused by previous viral infections and rapid uptake of artificial released proteins that are mimicking the structure of viral proteins.

It has been demonstrated that the arginine-rich motif (NoS) can bind RNA. However, the specificity of this binding is mediated by the few amino acids other than the arginine present within this stretch (43). It has been demonstrated for wild-type Rev that the specific RNA binding is mediated by the presence of an asparagine (43). Of note, this asparagine is also present in the NoS of the FXR proteins.

In conclusion, the strong sequence homology of the FXR proteins and their possible functional relationship with the viral protein Rev might shed light on RNA transport and regulation and improve our understanding of the complex molecular pathology of fragile X syndrome.

MATERIAL AND METHODS

DNA constructs

The expression vector containing the full-length FMR1 cDNA (pSF2) (9,53). A 2740 bp human FXR2 cDNA clone was cloned into the *Eco*RI site of the eukaryotic expression vector pSG5 under the SV40 promoter. The human FXR1 iso-d cDNA (17) was digested with *Eco*RI and *Bam*HI and cloned in the pSG5 vector. Murine *Fxr1h* iso-e and iso-f cDNA clones were generated as follows. An RT–PCR product generated from primers LLK186 and LLK192 and corresponding to iso-e was digested with *Bam*HI and *Eco*RI and cloned into the pcDNA3.1/HisB vector (Invitrogen, Groningen, The Netherlands). This vector puts His_6 and Xpress epitope tags in-frame with the N-terminal. This construct is known as *pHisB-iso e*. A 1400 bp *Bsm*I–*Eco*RI fragment from this construct (corresponding to the last two-thirds of the cDNA) and a 650 bp *Hin*dIII–*Bsm*I fragment from a full-length iso-d cDNA (26) were ligated into *Hin*dIII–*Eco*RI digested pcDNA3.1 to generate *pcDNA-iso e*. To generate *pcDNA-iso f*, the *Hin*dIII–*Pml*I fragment from *pcDNA-iso e* was replaced with the corresponding fragment from the iso-d cDNA (26). All three constructs, *pHisB-iso e*, *pcDNA-iso e* and *pcDNA-iso f*, were checked by sequencing

Cell culture, transfection and exportin1 inhibition

COS cells were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (FCS) at 37°C and 5% CO_2. The day before transfection the cells were seeded on glass coverslips. Transfections were performed as described by the manufacturer using 0.5 µg DNA, 3 µl Plus reagent and 2 µl Lipofectamine (Gibco BRL, Broda, The Netherlands). Cells were fixed for immunofluorescence 24 or 48 h after transfection.

Cells were treated for 3 h or overnight with LMB (50 ng/ml) to selectively block the function of exportin1.

Immunofluorescence labelling and detection

Cells were fixed for immunofluorescence 24 or 48 h after transfection in 0.1 M phosphate-buffered saline (PBS) containing 3% paraformaldehyde (pH 7.3) for 7 min at room temperature followed by a permeabilization step in 100% methanol for 20 min. Primary and secondary antibody incubations were performed for 60 min at room temperature in blocking buffer PBS pH 7.4 containing 0.15% glycine (Fluka, Zwijndrecht, The Netherlands) and 0.5% bovine serum albumin (Fluka). The primary antibodies were Ab1937 (1:200), directed against FXR2P and Ab2107 (1:200), directed against FXR1P. The fluorescent secondary antibodies labelled either with FITC or TRITC were used at 1:100 dilutions (DAKO, Glostrup, Denmark). Images were captured using the Power Gene FISH system on a Leica DMRXA microscope at ×1000 magnification. Images were processed using a filter wheel (Chroma Technology, Brattleboro, VT) and Adobe PhotoShop.

SDS–PAGE and western blotting

Protein samples in Laemmli buffer (54) were separated on either 10 or 7.5% SDS polyacrylamide gels followed by electroblotting on to nitrocellulose membrane (Schleicher & Schuell, Hertogenbosch, The Netherlands). Immunodetection was carried out using the mouse monoclonal antibody 1C3 against FMRP diluted 1:2500 (55), Ab2107 (1:4000) antibody against FXR1. The secondary antibody was coupled to peroxidase, allowing detection with the chemiluminesce method (ECL KIT, Amersham, Roosendaal, The Netherlands).

Peptide synthesis

A synthetic peptide C-PQRRNRSRRRRFRGQ representing the NoS (NoS2) as found in FXR1P was synthesized on a Nova Crystal Peptide synthesizer using solid phase and Fmoc chemistry. The peptide was purified and analysed by high-performance liquid chromatography and the amino acid sequence was analysed using a protein sequencer 473A from Applied Biosystems. The purified peptide was coupled to the fluorescent dye Cy3 (Amersham Life Science, Inc.). The synthetic peptide was added to the media (Ham's F10, Gibco BRL, supplemented with 10% FCS) of cultured fibroblasts, at a final concentration of 10 µg/ml. After 24 h the cellular uptake of the fluorescent peptide was monitored using the Power Gene FISH system on a Leica DMRXA microscope at ×1000 magnification. Images were processed using a filter wheel (Chroma Technology) and the Adobe PhotoShop software package.

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